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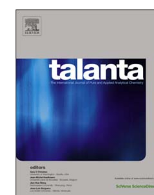

Identification and quantification of bleomycin in serum and tumor tissue by liquid chromatography coupled to high resolution mass spectrometry

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ABSTRACT

Bleomycin is a cytotoxic antibiotic available as a compost of structurally strongly related glycopeptides, which is *in vivo* found chelated with several metals. Its pharmacotherapy has merely been based on experimental dose – response data, whereas its biodistribution and pharmacokinetics remain fundamentally unknown. This is reasoned by an absence of a specific and sensitive mass spectrometry-based analytical method for its determination in biological tissues. We herein reveal the results of our study on the mass spectrometric behavior of two main bleomycin fractions A2 and B2, including their metal complexes, particularly the predominant copper chelates. In the electrospray ion source bleomycin forms double charged species, where for the metal-free fraction A2 and its copper complex *m/z* 707.76 and *m/z* 707.21 are seen, respectively. Hence, the second isotopic ion of the chelate (*m/z* 707.71) nearly coincides with the first isotopic ion of the metal-free fraction. This phenomenon can only be followed by high-resolution mass spectrometry, and is considered the plausible reason, why the attempts to determine bleomycin with mass spectrometry have been so scarce. The presented paper further describes a sensitive and selective liquid chromatography – mass spectrometry analytical method for determination of bleomycin in serum and tumor tissues. This newly developed method was employed for bleomycin pharmacokinetic studies in serum and tumors of laboratory animals. Additionally, the method was employed for determination of bleomycin pharmacokinetic parameters in elderly patients in order to determine the effective therapeutic window of electrochemotherapy with bleomycin.

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1. Introduction

Bleomycin (BLM) is a group of glycopeptide antibiotics produced by *Streptomyces verticillus*. It is now clinically used in the treatment of several neoplastic diseases including squamous cell carcinoma, non-Hodgkin's lymphoma, testicular carcinoma, ovarian cancer, *etc.* [1]. BLM is a mixture of more than ten components that differ one from another in their terminal amine moiety [2]. Bleomycins A1, demethyl-A2, A2, A2′-a, A2′-b, A2′-c, A5, A6, B1′, B2, B4 and BLM acid are well characterized [2,3]. Clinically administered BLM consists of 55–70% of BLM-A2 and 25–32% of BLM-B2 as the two most common constituents. The remaining percentage is divided among other subfractions [4].

The structurally complex bleomycins contain four distinct regions (Fig. 1) consisting of N-terminal domain, which is responsible for metal binding, oxygen activation and site-selective DNA cleavage. The N-terminal domain is connected via methylvalerate-threonine linker peptide to a C-terminal domain containing a bithiazole moiety, which provides the majority of the DNA-binding activity. A disaccharide moiety consists of gulose and mannose sugars connected to the metal-binding domain. The metal-binding domain binds redox-active transition metals, such as Fe, Cu, Co, Ni, Mn and Zn [1].

BLM is isolated as a blue equimolar Cu^{2+} complex [2]. The copper can be removed by precipitation with hydrogen sulfide in methanol (MeOH) solution to give a colorless metal-free BLM. The copper complex of BLM can be regenerated from metal-free BLM. That is, treatment of metal-free BLM with an excess amount of inorganic cupric salt in neutral aqueous solution regenerates the

Abbreviations: BLM, bleomycin; LLOQ, lower limit of quantification; QTOF, hybrid quadrupole coupled to TOF MS; BLM-A2-Cu, bleomycin A2 fraction copper complex; BLM-B2-Cu, bleomycin B2 fraction copper complex; MeOH, methanol; ACN, acetonitrile

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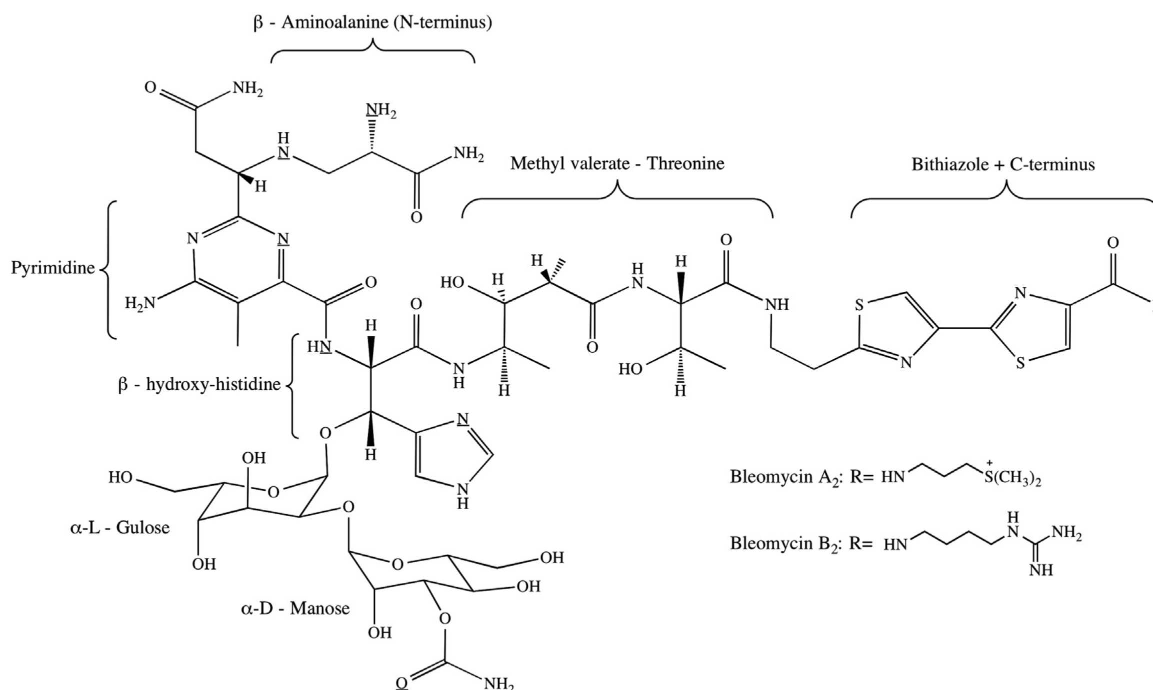

Fig. 1. Structures of BLM-A2 and B2 with four functional domains of the molecule (adapted from [1]).

original equimolar Cu^{2+} complex in an excellent yield. It is remarkable that only the natural copper-complex is regenerated almost quantitatively though there are many potential coordination sites in the molecule [2]. The copper complex and similarly other metal complexes of BLM have six coordination sites, of these four equatorial nitrogenous ligands including N^{π} from the imidazole of β-hydroxyhistidine, the deprotonated amide nitrogen of histidine, the pyrimidine ring nitrogen and the secondary amine of β-aminoalanine [1,2]. Further, the primary amine of β-aminoalanine binds axially, while the remaining axial coordination site is most likely occupied by carbamoyl oxygen, carbamoyl nitrogen, or dioxygen in case of activated BLM and it appears to be a dynamic structure [1,2]. The atoms involved in metal ion binding are underlined in Fig. 1.

An improved understanding of BLM pharmacological mechanism of action and its pharmacokinetics are of crucial importance for planning of different chemotherapeutic schedules or combined therapy such as electrochemotherapy in cancer patients, where timing of the applied permeabilizing electric fields with regard to intravenous BLM injection determines the efficacy of therapy. This is only possible by reliable identification and quantification of BLM in target biological tissues. Only few analytical methods have been developed before to determine BLM congeners, including their metal complexes or metabolites in biological matrices [5–11]. However, most of them employed less selective identification techniques such as UV absorption [5,6], radioimmunoassay [7] and fluorescence detection [8], which are unable to distinguish between clinically important components of BLM composite. Mahdadi et al. [8] developed an IP RP-HPLC method with fluorescence detection to determine BLM metabolites deamido-BLM-A2 and deamido-BLM-B2 in the plasma of patients undergoing BLM therapy and in rat hepatocytes that had been exposed *in vitro*. Unfortunately, the method sensitivity of 70 ng mL^{-1} was not sufficient to determine the two metabolites in actual human plasma samples, although the metabolic deamidation in rat hepatocytes was proved [8]. Mabeta et al. [9] successfully separated BLM-A2 and BLM-B2 fractions by using an IP RP-HPLC with UV absorption detection after a protein precipitation with MeOH. The method was validated, reaching the LOD of 0.1 and 0.2 µg mL^{-1} for BLM-A2 and BLM-B2, respectively [9]. Yet, the linearity was confirmed over narrow concentration ranges, so that the measured plasma levels of BLM-A2 and BLM-B2 exceeded the upper limits of quantification [9]. To the best of our knowledge, only one very recent study attempted to determine Cu complexes of BLM-A2 and B2 fractions in pharmaceutical preparations by using a selective and sensitive identification technique such as MS after a chromatographic separation [10]. Despite the research group [10] proposed that the analytical method could also serve for determination of BLM in plasma, only 1 µg mL^{-1} BLM-spiked plasma samples were tested, whereas no method validation was performed.

BLM is, besides cisplatin, the only cytostatic drug so far, which is employed in routine clinical use of electrochemotherapy for treatment of various cancer hystotypes in more than 140 oncological centers across Europe [12]. Electrochemotherapy is a treatment method that uses low-dose chemotherapeutics, but achieves up to 1000-times higher efficiency based on increased permeabilization of cancer cell membranes under applied electric field. This increased permeabilization enables larger quantities of BLM to enter the cells, where it acts primarily as endonuclease [12]. In this respect, the use of BLM is achieving new and not yet recognized dimensions and therefore it is very important to improve the knowledge on its pharmacokinetics. The determination of BLM serum levels can be used to calculate BLM dosage adjustments and will enable the determination of optimal interval between the injection of bleomycin and application of electric pulses for effective therapy, which was previously set only empirically [13]. The presented study reports on the development and validation of an UPLC coupled to high resolution MS method for determination of BLM. The analytical method was employed to follow BLM pharmacokinetics in serum and tumor tissues of test animals and oncological patients. In view of the limited studies performed in this field, we consider it is of high significance for the forthcoming research to elucidate the MS behavior of BLM and its metal complexes, as presented herein.

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